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Two-Way Chemical Signaling in *Agrobacterium*-Plant Interactions

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INTRODUCTION

Agrobacterium species are gram-negative, obligately aerobic soil bacteria capable of saprophytic or parasitic growth and are responsible for the crown gall and hairy root diseases of dicotyledonous plants (53). The genus contains two widely studied pathogenic species: *Agrobacterium tumefaciens* and *A. rhizogenes* (115). Both infect a broad variety of plants and infect only at wound sites. They infect individual cells at the site of infection and cause these cells to proliferate; *A. tumefaciens* causes either a gall of disorganized callus tissue or a teratoma containing stunted shoots, and *A. rhizogenes* causes a proliferation of morphologically distinctive roots. In each case, this is achieved by the transfer of a discrete fragment of bacterial DNA to the nuclei of plant cells, where it is integrated into genomic DNA and directs the overproduction of or hypersensitivity to plant growth hormones. This transferred DNA (T-DNA) also directs the production of novel compounds called opines, which provide a source of nutrients to the colonizing bacteria. The T-DNAs are localized prior to transfer on a family of large plasmids, called Ti (tumor-inducing) plasmids for *A. tumefaciens* or Ri (root-inducing) plasmids for *A. rhizogenes*. Ti and Ri plasmids also contain nontransferred genes which encode proteins that mediate T-DNA transfer and other nontransferred genes which direct opine catabolism. In addition to these diseases, some strains of *A. tumefaciens* can incite a root decay on susceptible grape vines (24). Since strains lacking the Ti plasmid can cause this disease, no DNA need be transferred during infection, but at least one degradative enzyme, a pectinase, is released by the bacteria.

Agrobacterium is the only genus of bacteria that is thought to transfer DNA to higher organisms as a normal part of its behavioral repertoire. However, one of the more surprising and rewarding aspects of recent developments in the field is the finding that this ability most probably evolved from other, more widely distributed kinds of bacterial metabolism (208). First, the transfer of DNA probably evolved from a conjugal transfer system. Second, every *Agrobacterium* signal transduction system so far studied that is required to perceive plant-released molecules is homologous to some other bacterial regulatory system. Understanding how *Agrobacterium* spp. perceive wounded plants and transfer DNA to them has been aided immeasurably by uncovering and exploiting the similarities between this system and homologous bacterial systems. Just as students of *Agrobacterium* spp. have benefitted from developments in other fields of molecular biology, they have made a number of discoveries that are of interest to a wider audience. The notion that plant-associated bacteria might induce the genes required for association in response to host-released signal molecules originated with studies of *A. tumefaciens*. This phenomenon was later found to be true of *Rhizobium* species as well as a variety of other plant and animal pathogens (148). The regulatory system that controls this induction is arguably one of the best characterized of its gene family. A second example involves the mechanism of DNA transfer. Assuming that this transfer is essentially a type of bacterial conjugation, it arguably has been more completely characterized than any other conjugal transfer system.

In this review I will attempt to describe various aspects of *Agrobacterium*-plant interactions and will place special emphasis on the exchange of chemical signals during infection. The word "signal" is taken in its broadest sense to denote any compound (including diffusible chemicals, polysaccharides, proteins, and DNA) which plays a role in this plant-microorganism interaction. The first section will deal with plant-released compounds that are perceived by *Agrobacterium* spp., and the second section will analyze signals transferred from the bacterium to the plant, especially the DNA which is transferred from bacteria to plant nuclei. This study also will strongly emphasize recent findings, including unpublished work presented at recent symposia and elsewhere. This rapidly moving field of research has been the subject of many other recent reviews: some have emphasized signal exchange (20, 52, 58, 83, 153, 154, 249, 258a); others have treated general aspects of crown gall tumorigenesis (16, 21, 75, 94, 109, 157, 196, 253, 258, 270–272) or mechanisms of T-DNA transfer (119, 173, 208, 209); and still

others have discussed the use of *Agrobacterium* spp. in creating transgenic plants (72, 118, 126, 249–251).

PLANT-RELEASED SIGNAL MOLECULES

Chemotaxis and Attachment

Agrobacterium spp. are peritrichous motile organisms, and there are several reports indicating that motility and chemotaxis play a role in the early events of infection. In fact, it seems a priori quite probable that chemotaxis would have to play an important role in infection, since without it the cell-cell contact which is required for DNA transfer would rarely occur. Hawes et al. (85) developed assays to measure translocation toward excised root tips and isolated root cap cells. Several wild-type strains containing or lacking the Ti plasmid exhibited chemotaxis toward excised root tips from all plant species tested and toward root cap cells of pea and maize. Strain A348, taken as representative, was also attracted to many but not all amino acids and sugars. Transposon insertion mutants deficient in chemotaxis were obtained. Among the strains isolated were (i) nonmotile or poorly motile strains; (ii) two strains which were motile but not attracted to root tissues or cells, sugars, or amino acids; (iii) one strain which was attracted to sugars and amino acids but not to plant tissues or cells; and (iv) one strain which was attracted to root tips but not to excised cells. In a subsequent study, four of these mutants were assayed for the ability to form tumors on pea plants (84). When used to inoculate plants directly, they were fully virulent, but when they were used to inoculate soil, which was dried and then used to grow plants, the nonchemotactic mutants were completely avirulent. However, in similar treatments in which sand was used in place of soil, these strains were almost as virulent as the wild-type strains. These results suggest that chemotaxis is critical in certain soil types, but may be less important in other, less compact soils.

Which chemoattractants are likely to be important in drawing *Agrobacterium* spp. to plant wounds? Well-aerated soil tends to be poor in utilizable growth substrates, whereas root exudates and especially wound exudates release large amounts of these materials (114). It seems possible that the bacteria are drawn to any of a large number of wound-released compounds, including amino acids and sugars. However, several reports from one research group (11) argue that *A. tumefaciens* C58 is attracted to a group of phenolic compounds previously identified as *vir* gene inducers (see below). Acetosyringone and related compounds elicited chemotaxis in two different assays. The range of concentrations active in chemotaxis was quite narrow, with a peak of 100 nM. Chemotaxis required the Ti plasmid, and specifically the regulatory genes *virA* and *virG* (189). However, these results have been difficult to reproduce in at least two other laboratories. One group failed to detect chemotaxis toward acetosyringone at any concentration (84), whereas another group also reported that acetosyringone did not elicit chemotaxis and that chemotaxis toward related compounds did not require the Ti plasmid (162). However, Ashby et al. have described the strain used by these other groups (strain A348) as being weakly motile compared with strain C58 (11). Nevertheless, strain A348 is attracted to other components of wound exudate such as a variety of sugars. It does seem difficult to rationalize a role for VirA and VirG in chemotaxis, given what is known about their roles in transcriptional regulation (see below). Although it is true that these proteins are distantly related to several proteins involved in chemotaxis, they are much more closely related to a family of regulatory proteins which have no direct role in chemotaxis.

One final point about chemotaxis is that these reports are similar to observations about *Rhizobium meliloti*. Gaworzewska and Carlile (74) reported that *R. meliloti* exhibit chemotaxis toward root exudates, whereas Caetano-Anolles et al. (25) reported a very low level of chemotaxis toward luteolin, a compound which is known to induce the transcription of genes required for nodulation (168). Mutations in *nodA*, *nodC*, or *nodD* abolished chemotaxis toward luteolin. Chemotaxis-deficient mutants nodulated plants poorly when small numbers were used to inoculate soil (26).

A second early step in infection is the binding of bacteria to target plant cells. Specific receptors may exist on the bacterial and plant cell surface, since (i) binding of *A. tumefaciens* to plant cells is saturable and (ii) several other genera of bacteria are not able to compete for binding sites (127). A number of bacterial strains with mutations reported to affect this step have been isolated. All such mutations are chromosomal, and no known *vir* mutation affects binding (62, 121).

Three chromosomal genes, *chvA*, *chvB*, and *exoC*, are required for the synthesis of a cyclic β-1,2-glucan which has been implicated in plant cell binding. Mutations in *chvA*, *chvB*, or *exoC* strongly attenuate virulence (29, 62, 112, 220) and cause a 10-fold decrease in binding of bacteria to zinnea mesophyll cells (62). ChvB is involved in biosynthesis of the glucan (275), while ChvA appears to be required for the export of this polysaccharide from the cytoplasm to the periplasm and extracellular fluid (30, 55). *chvA* is homologous to a family of membrane-bound ATPases involved in active transport (30). The cyclic β-1,2-glucan has been implicated in resistance of the bacteria to low osmotic pressure (149), and, indeed, *chvA* or *chvB* mutants grow more slowly than wild-type cells in hypoosmotic broth. However, these strains are avirulent in media with either high or low osmotic pressure (31). Genes homologous to *chvA*, *chvB*, and *exoC* have been identified in *R. meliloti*; strains containing mutations in these genes are unable to form effective root nodules (29, 64, 65). A cautionary note, however, is that *chv* and *exoC* mutants are pleiotropic, exhibiting deficiencies in polysaccharide production, conjugal transfer of some but not all plasmids, and motility. Although *chv* products do appear to be mechanistically involved in β-1,2-glucan biosynthesis, it is not clear whether the polysaccharide itself is involved in attachment or whether it affects some other property of the cell surface which is directly involved.

Two other classes of chromosomal genes have been described as encoding proteins which are also important in attachment. The *cel* product is important in cellulose biosynthesis. Strains with mutations in *cel* still bind to plant cells, but do so individually rather than forming aggregates (135). These mutants are still virulent, but are more susceptible to being washed from carrot cells than are wild-type bacteria. Other mutants of *A. tumefaciens* and *A. rhizogenes* showing a deficiency in attachment were obtained by screening mutagenized cultures directly for attachment deficiency (49, 136). All the binding-deficient mutants obtained were avirulent, although the extremely high frequency at which these mutants were isolated is difficult to reconcile with the much lower frequencies at which avirulent Tn*5* mutants have been obtained by others (73). It is also surprising that the mutants were unstable and readily reverted to the wild-type

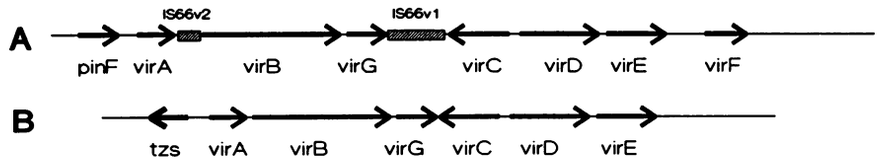

FIG. 1. Genetic organization of the *vir* region of the octopine Ti plasmids (A) and nopaline Ti plasmid (B). Arrows indicate transcriptional units. Insertion sequences IS*66*-V1 and IS*66*-V2 are described in reference 130

phenotype. The cloned *A. rhizogenes* attachment genes did not hybridize to cloned attachment genes from *A. tumefaciens*. The *A. tumefaciens* attachment mutants lacked a number of outer membrane proteins.

vir Gene Induction

Approximately 25 Ti plasmid-encoded *vir* genes, arranged in either six or seven operons (Fig. 1; Table 1), are required for tumorigenesis. Most of these gene products are mechanistically involved in the processing of the transferred DNA, as described below. All Ti plasmid-encoded *vir* operons are transcriptionally induced during infection by a family of related phenolic compounds and also by a family of sugars, some of which are released from plant wounds (6a, 28, 178, 203, 204, 263). Transcriptional induction was first observed by cocultivating *A. tumefaciens* with isolated plant cells, cultured tissues, or protoplasts (204). Stachel et al. later isolated two potent inducers, acetosyringone and hydroxyacetosyringone, from tobacco cells or roots (202). Several other groups tested a large number of related compounds for inducing activity. Bolton reported that a mixture of seven phenolic compounds, when added in combination, caused induction (17). However, two other groups retested each of these compounds and found that one of them, vanillin, was active, while the other six were inactive (143, 200). These groups also tested a large number of synthetic phenolics and reported that many of them were active, including sinapinic acid, guaiacol, and many other structurally similar compounds. Recently, a phenylpropanoid glucoside called coniferin, isolated from the gymnosperm *Pseudotsuga menziesii* (Douglas fir), was also shown to induce *vir* genes (152). *Agrobacterium* strains which were more tumorigenic on this plant were more efficiently induced by coniferin than were other less tumorigenic isolates. A bacterial β-glucosidase was postulated to be required to convert this compound to coniferyl alcohol.

TABLE 1. Descriptions of the *vir* genes of the octopine and nopaline Ti plasmids

Locus	Size (kb)	No. of genes	Function	References
virA	2.8	1	Environmental sensor	104, 125, 142–144, 150, 151, 264
virG	1.0	1	Transcriptional activator	103, 105, 164, 165, 171, 261
virC	1.5	2	T-DNA processing	47, 227, 228, 267
virD	4.5	5	T-DNA processing (*virD1* and *virD2* encode T-DNA border endonuclease)	60, 63, 88, 95, 101, 244, 268, 269
virE	2.2	2	T-DNA processing (*virE2* encodes ssDNA-binding protein)[a]	42, 44, 50, 79, 91, 260
virB	9.5	11	Transmembrane pore?	123, 192, 222, 245, 246
virF	1.0	1	?	141, 160
pinF	2.8	2	Cytochrome P-450 monooxygenases	113
tzs	1.0	1	Cytokinin biosynthesis	106, 174

[a] ssDNA, single-stranded DNA.

Specific monosaccharides also play a central role in *vir* gene induction. Glucose, galactose, arabinose, fucose, and xylose all strongly potentiated induction, as did 2-deoxy-D-glucose and 6-deoxy-D-glucose, which are not catabolized (28, 191). Arabinose and fucose allowed some induction of a *virE-lacZ* fusion even in the absence of phenolic inducers (28). In a subsequent study, other monosaccharides and similar compounds were tested; only sugars with equatorial hydroxyls at C-1, C-2, and C-3 positions were active, while C-4 could be either epimer, and a wide variety of substitutions at C-5 were permissible (8). Ketoses and most disaccharides were inactive, although cellibiose was active (28, 191). The most potent inducers were the acidic sugars D-galacturonic acid and D-glucuronic acid. Most of these compounds are monomers of plant cell wall polysaccharides and were therefore postulated to be exuded from a wide variety of plant wounds. Induction has been reported to be potentiated by a variety of opines, which are normally released by plant tumors (see below) (234).

A third environmental signal of central importance in *vir* gene induction is extracellular pH. Induction does not occur at neutral pH and appears most efficient at extremely low pHs (in the range of 5.0 to 5.5), which are inhibitory to cell growth (204). The underlying mechanism by which pH influences induction is not clear. However, the hypothesis that it is attributable to the pH-inducible promoter of *virG* (see below) was disproved by expressing *virG* from the *lac* promoter and showing that the acidic pH optimum was not affected (37). The natural osmoprotectant glycine betaine offered some protection from the toxic effects of acidic media and thereby increased *vir* induction (235).

The rather broad host range of most *Agrobacterium* strains can be explained in part by the identification of *vir* gene inducers. The compounds described above are present at wound sites of a broad range of host plants. First of all, most plant wounds are acidic, since most plant vacuoles are acidic (110). Similarly, phenolic and monosaccharide inducers are probably general features of most plant wounds. This is because certain phenolics (including coniferyl alcohol and sinapyl alcohol) are needed to make lignin, whereas certain neutral and acidic monosaccharides are needed to make cell wall polysaccharides. These two plant cell wall polymers are actively synthesized at most wound sites (110). It makes perfect sense for *Agrobacterium* species, most of which infect a broad range of plants, to recognize compounds that are common to all of them. This situation stands in stark contrast to the induction of *Rhizobium* nodulation genes by plant-released flavones (128). In this case, each *Rhizobium*

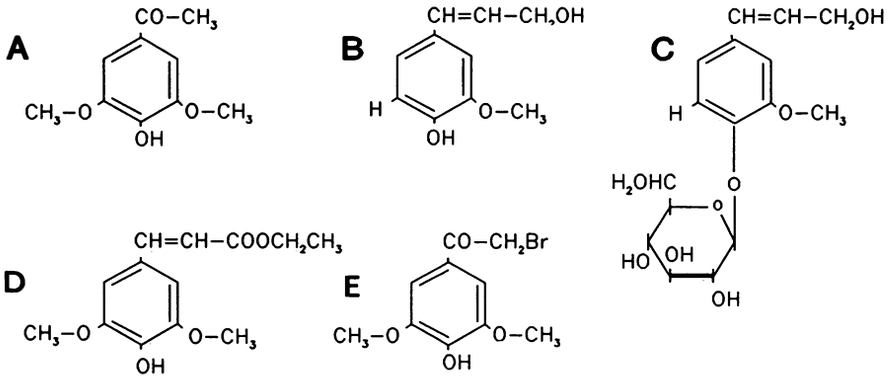

FIG. 2. Chemical structures of a representative group of phenolic compounds which are active in induction of the *vir* regulon. (A) Acetosyringone; (B) coniferyl alcohol; (C) coniferin; (D) ethyl ferulate; (E) bromoacetosyringone, an inhibitor of induction.

species infects only one or a few species of legumes. Each host species releases a unique flavone, which is recognized by its cognate *Rhizobium* species but not by other *Rhizobium* species.

The finding that host plants release *vir* gene inducers led to speculation that nonhost plants, including virtually all monocots, might prevent infection simply by not releasing these compounds. Supporting this hypothesis are two reports that preinduction of *A. tumefaciens* by acetosyringone or by wound exudates extends its host range (161, 184). Furthermore, it was reported that exudates from seedlings of seven genera of monocots failed to release *vir* gene inducers, although homogenates of wheat seedlings did contain inducers (230, 231). However, workers in a different laboratory assayed conditioned media from suspension cell cultures of five monocot species (maize, wheat, barley, rice, and asparagus [146]). Wheat cells released high levels of inducing compounds, while maize, rice, and asparagus released lower levels and barley did not release detectable inducers. The compound released from wheat cells was identified as ethyl ferulate, which is a somewhat more potent *vir* gene inducer than the widely used acetosyringone. If it is true that plant wounds of nonhosts release *vir* gene inducers, then the block that prevents infection of nonhosts is probably seldom at the level of *vir* gene induction (see also reference 81).

One observation that may complicate the search for inducers is the finding that an antibacterial metabolite from corn seedling homogenates strongly inhibits growth and *vir* gene induction (182). Therefore, the inability to detect inducers may be due to the presence of compounds that interfere with induction. In fact, an analog of acetosyringone, bromoacetosyringone, has been identified as a specific inhibitor of *vir* gene induction (90). Figure 2 shows the structure of this inhibitor and of several inducing compounds.

Proteins Which Mediate *vir* Gene Induction

Induction of *vir* genes requires two plasmid-encoded proteins, VirA and VirG; mutations in the genes encoding either of these proteins completely block the response to plant phenolics (178, 207, 263). Potentiation of induction by monosaccharides requires a third protein called ChvE, which is chromosomally encoded (97). VirA and VirG are members of a gene family of two-component regulatory systems (125, 144, 145, 150, 171, 261). VirA is a member of the histidine protein kinase class, some of whose members have been proven to be kinases. VirG is a member of the response regulator class of proteins, whose N-terminal halves are the targets of phosphorylation and whose C-terminal halves generally have promoter-binding properties (reviewed in references 4, 148, and 213).

Octopine-type Ti plasmids encode a VirA protein of 829 amino acids, which has a typical leader sequence, suggesting that at least a portion of the protein crosses the cytoplasmic membrane. About 250 amino acids toward the C terminus is a second hydrophobic region (amino acids 261 to 280), followed directly by the positively charged sequence Arg-Leu-Arg-Lys-Lys (125, 144). This resembles a number of stop-transfer sequences in other membrane-spanning proteins (122). The VirA proteins encoded by the C58 and Ag162 plasmids appear quite similar (125, 150). The hypothesis that VirA spans the cytoplasmic membrane was confirmed by using Tn*phoA* to create gene fusions between *virA* and *phoA* (142, 264). Confirmatory evidence was obtained by first converting an *Escherichia coli* culture expressing VirA to spheroplasts, then exposing the spheroplasts to proteolysis to digest extracellular proteins, and then analyzing the remaining proteins by Western immunoblotting with a VirA antibody (264). Protease treatment removed about 270 amino acid residues from the N terminus.

Recently, data from several groups have confirmed the hypothesis that VirA can undergo autophosphorylation (98, 104, 151). In one study, fragments of VirA were overproduced by deleting the promoter-proximal 158, 967, or 1,240 bp and fusing the remaining fragment of the gene to the 5′ end of *lacZ* (104). The VirA fragments thus generated formed inclusion bodies in *E. coli* and were gel purified and renatured. Upon incubation with [γ-^{32}P]ATP, the γ-phosphate was transferred covalently to VirA. Neither [γ-^{32}P]GTP nor [α-^{32}P]ATP transferred labeled phosphate. The phosphate was bound to a histidine residue. Only one histidine residue is absolutely conserved among homologous proteins. The codon encoding this residue (amino acid 474) was altered by site-directed mutagenesis to a glutamate. The mutant *virA* gene was nonfunctional at directing *vir* gene induction in vitro, and the same mutation in an overproduced VirA fragment prevented autophosphorylation in vivo (104). However, in other studies, a TrpE-VirA fusion protein which lacked the first 524 amino acid residues of VirA, including its conserved histidine, was constructed (98, 151). This fusion protein was also able to carry out autophosphorylation.

Phosphorylated VirA fragments can transfer phosphate to VirG in vitro, even after removal of [γ-^{32}P]ATP (103). The phosphate is attached to aspartate residue 52. This residue is absolutely conserved among homologous proteins and is known to be the site of phosphorylation of CheY and NtrC (213). Alteration of this aspartate to asparagine abolished the ability of the protein to accept phosphate from VirA in vitro and resulted in a nonfunctional VirG protein in vivo (179). Although not demonstrated, phosphorylation is presumed either to alter the ability of VirG to bind *vir* promoters (see below) or to alter its ability to contact RNA polymerase.

VirG protein has been postulated to bind to a family of similar sequences, called *vir* boxes, located upstream of each *vir* promoter (212, 262). A similar but not identical family of sequences are also found upstream of VirG-inducible promoters of *A. rhizogenes* (9). These sites are often but not always present in multiple copies at 11-bp intervals. Deletion or alteration of either of two *vir* boxes located in the *A. tumefaciens virG* gene abolished induction of that promoter (257). Similar data were obtained when *vir* boxes were altered or deleted in the *virB*, *virE*, *virC*, and *virD* promoters (51, 105, 163, 164). Direct binding of VirG protein to these *vir* boxes in vitro has been demonstrated for the *virB*, *virE*, and *virG* promotes of *A. tumefaciens* (150, 163, 164, 170, 172) and the *virC* and *virG* promoters of *A. rhizogenes* (216). A fragment of VirG consisting solely of the carboxy-terminal half of the wild-type protein was able to bind these sites, indicating that DNA binding is carried out by this half of the protein (170, 179). In all these studies, VirG was isolated from *E. coli* strains and is therefore presumed to be nonphosphorylated. It is not known which properties of VirG are changed by phosphorylation, but studies of OmpR, a closely homologous regulatory protein, suggest that phosphorylation may increase the affinity of VirG for its binding sites (1).

The transmembrane topology of VirA suggests that it may be an environmental sensor, able to recognize either directly or indirectly the phenolic compounds and monosaccharides that cause *vir* gene induction. This proposed role is in accord with that of the homologous proteins (213), although experimental evidence that these proteins truly are sensors is limited to the *R. meliloti* FixL protein, which is oxygen responsive in vivo and in vitro (80). It was postulated that the periplasmic domain of VirA might contain the binding sites for these inducers, and therefore it was surprising that a deletion of most of the periplasmic domain of VirA did not abolish responsiveness to acetosyringone (142). Deletion of this domain did, however, alter the normal acidic pH (less than 5.5) and low-temperature (29°C) optima of the wild-type protein. Assuming that VirA itself does bind acetosyringone at all, the binding site must lie either in the membrane-spanning regions or in the cytoplasmic portion of the protein. *virA* mutants which activate *vir* gene transcription even in the absence of inducers have been isolated (7, 165). One of the mutations isolated by Pazour and Das (165) was localized to the amino-terminal transmembrane domain; this codon was further mutagenized, and it was found that a basic amino acid did not abolish the function of the protein. Surprisingly, an additional basic amino acid in the transmembrane region also did not abolish protein function. This finding could be interpreted to mean that secretion of the protein is not required for its ability to carry out phenolic-responsive *vir* gene activation. Recent studies from my laboratory indicate that the entire periplasmic domain and the two transmembrane regions can be removed without disrupting phenolic-responsive *vir* gene activation (259), indicating that the binding site for phenolics must lie elsewhere.

An unusual feature of VirA is that its C terminus contains a domain homologous to the receiver domain of VirG (213). Similar receiver domains are found in a small number of homologous histidine protein kinases (10, 100, 140, 214). The function of this domain is unknown. Deletions removing part of it render the protein nonfunctional (142), but a deletion removing the entire domain had the opposite effect, resulting in increased levels of *vir* gene expression (259). It is tempting to speculate that this domain might be phosphorylated either by VirA itself or by some other kinase of the same gene family. Such a phosphorylation might even be environmentally regulated. Taken together, these data suggest that VirA must contain at least four domains (Fig. 3).

As noted above, efficient induction requires a third protein: that encoded by the chromosomal *chvE* gene (97). *chvE* mutants are extremely attenuated for *vir* gene induction and for virulence, resulting in a restricted host range. The reduction in induction can be partially overcome by using high concentrations of acetosyringone. The ChvE protein is homologous to a family of periplasmically localized sugar-binding proteins involved in chemotaxis and active transport. This potentiation of induction absolutely requires the periplasmic domain of VirA in addition to ChvE (28, 191). A model for the function of VirA, VirG, and ChvE is shown in Fig. 3.

A subset of *vir* genes appears to be responsive to additional regulatory systems. *virG* is one example. It has two promoters and is responsive to three environmental stimuli (207, 233, 257). One promoter is induced by acetosyringone in strains containing VirA and VirG proteins (257). In this sense, *virG* expression is identical to all other *vir* genes. However, this same promoter is also induced by phosphate starvation (257). This induction does not require VirA or VirG or any other Ti plasmid-encoded genes. Confirmatory evidence was obtained from studies of the *virG* gene of *A. rhizogenes*; this gene is transcribed from three promoters, and one of these is inducible by either acetosyringone or phosphate starvation (9). The phosphate starvation-inducible promoters of both genes bear a strong resemblance to the family of *E. coli* promoters which are induced by phosphate starvation (243). It has been proposed that *Agrobacterium* spp. have a regulatory system similar to the *pho* regulatory system of *E. coli* and that this system regulates *virG* (257). Finally, a second promoter, 50 bp downstream from the first, is induced by a family of environmental stresses, including extremes of pH, DNA-damaging agents, and heavy metals (133). This induction also requires no Ti plasmid-encoded genes. This promoter has a strong sequence similarity to the family of heat shock promoters of *E. coli*, but appears not to be strongly activated by either heat or ethanol. A mutation in the chromosomal *chvD* gene was reported to result in attenuated induction of *virG* by acidic pH or by phosphate starvation (263). However, in light of the more recent finding that the responses to these stimuli occur at different promoters, it seems more likely that the *chvD* mutation acted in an indirect fashion. It is believed that activation of *virG* by phosphate starvation or other environmental stresses may play an important role in the initial stages of induction, in order to increase the concentration of VirG protein to sufficient levels to allow autoregulation to function efficiently (257). This pump-priming expression of VirG would occur only under conditions of environmental stress, which would suggest that environmental stress should potentiate *vir* gene induction.

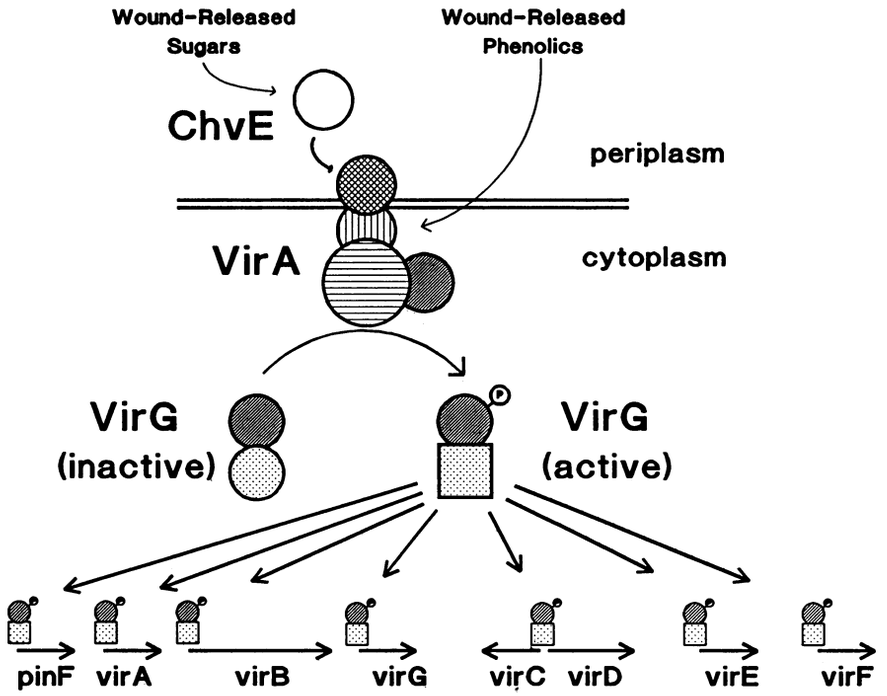

FIG. 3. Model describing the proposed function of the regulatory proteins VirA and VirG. VirA is a transmembrane protein kinase that may directly bind phenolic inducers, and VirG is a target of the VirA kinase and binds to *vir* promoters to activate their transcription. Reprinted with permission from reference 258a.

The regulation of *virC* and *virD* also seems to be distinguishable from that of other *vir* genes, in that they alone are transcribed at elevated levels in strains containing a mutation at the chromosomal *ros* locus (45, 46). This mutant was recovered by selecting elevated expression of a *virC*::*cat* fusion. The mutant also results in elevated expression of *virD*, whose promoter is close to and divergent from that of *virD*, but it does not affect transcription of *virB*, *virE*, *virG*, or *pinF* (also referred to as *virH*). *virC* and *virD* promoters are also expressed at high levels in *E. coli*, suggesting that a negative regulatory element may control expression in wild-type *Agrobacterium* strains. Recently, the *ros* gene was cloned and sequenced (48). This gene appears to be autoregulatory and to contain a site in its promoter which is also found in the *virC-virD* regulatory region. The Ros protein binds specifically to the *virC-virD* regulatory region (48). This regulatory system has been identified by mutation only, as no treatment which acts through *ros* to derepress *virC* and *virD* has been found. If such a treatment does exist, it would mean that these genes can be induced without other *vir* genes being induced. The proteins encoded by the *virD* operons are sufficient to catalyze nicks at the T-DNA borders (see below). It is of note also that *virE* (which, as described below, encodes a single-stranded DNA-binding protein) is expressed at a rather high basal level compared with other *vir* genes (203). Taken together, these observations mean that the postulated "T-complex" consisting of a linear single-stranded T-DNA complexed with VirD2 and VirE2, could be formed without the induction of any other *vir* gene. The adaptive significance of this is not clear.

Plant-Released Inducers of Chromosomal Genes

When an *Agrobacterium* strain lacking a Ti plasmid was cultured in medium containing a carrot root extract, induction of no fewer than 10 proteins was detected by one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Expression of one protein was inhibited by this extract (180). This strain was mutagenized with a transposon that generates *lac* fusions, and a derivative which responds to carrot root extract by induction of β-galactosidase was isolated. When this fusion was crossed into a strain containing a Ti plasmid, the resulting strain was fully virulent but tended to aggregate more than wild-type cells. The carrot inducer turned out to be the acidic polysaccharide pectate. Pectate has been shown previously to induce various *Erwinia* genes which encode pectinases (175). However, the strain used in this study probably does not encode a pectinase (although other biovars do make pectinases [177]). The possibility that a family of chromosomal genes is inducible by plant-released compounds should be addressed.

Signal Molecules Released by Plant Tumors

All known tumorigenic *Agrobacterium* strains are able to direct host plant cells to synthesize derivatized amino acids or sugars known as opines (217). Most strains transfer genes directing the synthesis of more than one of these compounds. Almost without exception, a strain which directs the synthesis of a particular opine has a corresponding nontransferred gene able to direct the catabolism of the same opine (218). At least 20 of these compounds have been described, although most are members of one of four families (67, 169). Figure 4 shows the structure of some representative examples of opines, and Table 2 provides a somewhat more comprehensive list. Although the opine hypothesis predicts that opine utilization provides a selective advantage over other bacteria in the rhizosphere (169), the ability to catabolize opines is not in fact limited to *Agrobacterium* spp., since

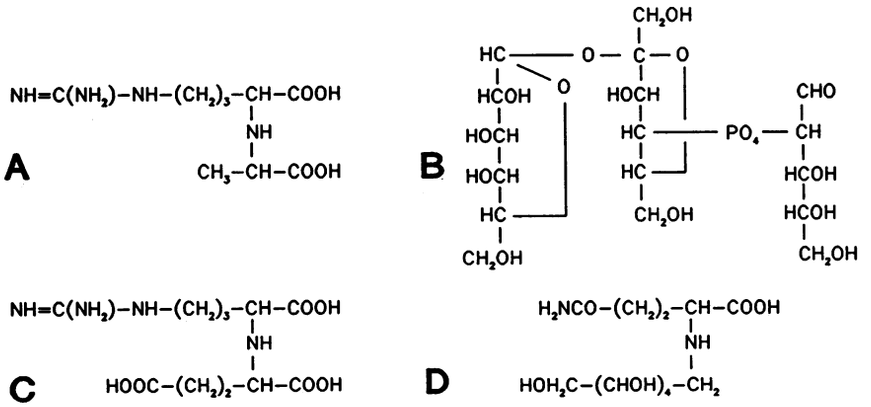

FIG. 4. Chemical structure of representative opines. (A) Octopine; (B) agrocinopine A; (C) nopaline; (D) mannopine. Synthesis of octopine and mannopine is directed by the Ti plasmids pTiA6 and pTi15955, while synthesis of nopaline and agrocinopines A is directed by the C58 and T37 Ti plasmids, among others.

various *Pseudomonas* and *Rhizobium* isolates are also able to do so (14, 20).

Several (perhaps all) opines induce the transcription of bacterial genes needed for their transport and catabolism. Recently, the regulatory proteins controlling the octopine, nopaline, and agrocinopine A/B catabolic operons have been mapped and sequenced. *occR* and *nocR*, which regulate the octopine and nopaline degradation operons, are members of the LysR family of transcriptional activators (82, 238). The product of *occR* negatively regulates its own gene and activates the transcription of the catabolic operon (82). Recently, OccR was purified and shown to bind to a specific site near the *occR* and *occQ* promoters (259). The region protected was slightly altered by the addition of octopine, indicating a direct interaction between octopine and OccR. The octopine and nopaline operons contain genes which are homologous to a family of four-component, "shock-sensitive" amino acid transport systems and therefore probably are involved in octopine transport (232, 238). The regulator of the agrocinopine catabolism operon, *accR*, is a member of the FucR family, and is, as predicted, a repressor (13). The genes required for degradation of mannityl opines have also been mapped. The four opines of this family appear to be transported and degraded by overlapping and partly redundant sets of genes (56, 57, 59, 71).

In addition to serving as a source of carbon, nitrogen, and sometimes phosphorous, a subset of opines, known as conjugal opines, are transcriptional inducers of Ti plasmid conjugal transfer genes (68, 117). Octopine induces conjugal transfer of the octopine-type plasmids, whereas agrocinopine A/B induces transfer of the nopaline-type plasmids. Older studies suggested that in both cases, opine degradation and conjugal transfer were regulated by a common regulatory system (68, 117). These regulatory systems were concluded to act by transcriptional repression, although for octopine-type plasmids, those findings are difficult to recon-

TABLE 2. Structure and occurrence of representative opines

Name	Substituents	Representative plasmids
Octopine family		
Octopine	Arginine, pyruvate	A6, R10, 15955, B6, Ach5
Octopinic acid	Ornithine, pyruvate	A6, R10, 15955, B6, Ach5
Histopine	Histidine, pyruvate	A6, R10, 15955, B6, Ach5
Lysopine	Lysine, pyruvate	A6, R10, 15955, B6, Ach5
Nopaline family		
Nopaline	Arginine, α-ketoglutarate	C58, T37, H100, K57
Nopalinic acid	Ornithine, α-ketoglutarate	C58, T37, H100, K57
Succinamopine	Asparagine, α-ketoglutarate	EU6, 181, T10/73
Leucinopine	Leucine, α-ketoglutarate	542, 398, A2, AT1
Mannopine family		
Mannopine	Glutamine, mannose	A6, R10, 15955, B6, Ach5
Mannopinic acid	Glutamic acid, mannose	A6, R10, 15955, B6, Ach5
Agropine	Mannopine lactone	A6, R10, 15955, B6, Ach5
Agropinic acid	Mannopine lactam	A6, R10, 15955, B6, Ach5
Agrocinopine family		
Agrocinopine A	Sucrose, arabinose	C58, T37, H100, K57
Agrocinopine B	Fructose, arabinose	C58, T37, H100, K57
Agrocinopine C	Sucrose, glucose	A4, 15834, 8196
Agrocinopine D	Fructose, glucose	A4, 15834, 8196

cile with the recent characterization of these regulatory systems described above. The fact that opines induce more than just their own catabolism genes means that they ought properly to be considered signal molecules. It is possible that additional genes are inducible by opines, and these are currently being sought in my laboratory. It would make sense that any protein which is needed by *Agrobacterium* spp. only when living in association with tumorous plant cells (for example, proteins required for colonization) would be regulated by opines.

Compounds similar to opines have been found in *R. meliloti* (155, 156). This organism fixes nitrogen in a mutualistic association with leguminous plants (128). Bacteria colonize roots of these plants and terminally differentiate into forms called bacteroids. These bacteroids synthesize and secrete derivatized sugars called rhizopines, which are catabolized by their soil-dwelling relatives. In this way, bacteria inhabiting a nodule can provide nutrients to free-living bacteria outside the nodule (155, 156). These findings may suggest a possible evolution of the opines in *Agrobacterium* spp.

SIGNAL MOLECULES RELEASED BY BACTERIA

Processing and Transfer of T-DNA

So far, we have considered the transfer of chemical messages from plant to bacterium. In return, *Agrobacterium* spp. release various chemical signals to plants. Of these, the most important and best understood consists of one or two discrete fragments of DNA called T-DNA, which are transferred from the Ti plasmid to plant nuclei (38, 39, 255). T-DNA must in some way be processed within bacteria to a transferrable form, traverse bacterial membranes and peptidoglycan, enter the plant cell cytoplasm and nucleoplasm, and become integrated into genomic DNA. I will begin by considering the *cis*-acting sites required for T-DNA transfer and then consider the roles of the various proteins that are required for transfer.

The junctions between the transferred and nontransferred DNA contain imperfect 25-bp direct repeats (266, 274). The right border is essential for efficient tumorigenesis and acts in a polar fashion, directing the transfer of sequences to its left (188, 241). In contrast to the right border, the left border is dispensable for tumorigenesis (107). A second sequence, called *overdrive*, is located near the right borders of both the T_L-DNA and the T_R-DNA of the pTiA6 and pRiA4 plasmids and near the right border of the pTiAB3 plasmid. The sequence near the T_L-DNA is needed for efficient tumorigenesis (166, 167). *overdrive* can be moved away from the right border at least 5 kb in either direction, and its orientation can be inverted without disrupting its function. Although no sequence similar to *overdrive* is found in nopaline-type plasmids, sequences flanking the right border enhance transfer efficiency (239), suggesting that some site functionally similar to *overdrive* may be present. In contrast, sequences flanking the left border inhibit transfer efficiency.

Upon induction of the *vir* regulon, a number of striking alterations of the T-DNA have been observed. Single-stranded scissions occur on the bottom strand at identical positions between bp 3 and 4 from the left end of each border (5, 101, 242). Double-stranded breaks have also been reported at this position (210, 233), although shearing of nicked DNA during isolation remains a possibility. In the octopine-type strain pTiA6, which has two T-DNAs and four borders, nicking was observed at each border (205, 233, 268). *virD1* and *virD2*, when expressed in *E. coli* from a foreign promoter, are sufficient to catalyze site-specific nicking at a border supplied in trans (60, 101, 268). The overproduction of VirD1 and VirD2 resulted in an enhanced level of T-DNA nicking and plant tumorigenesis (240). The characterization of these proteins in vitro is in its infancy, but one report indicates that VirD1 is a topoisomerase (76).

A large portion of the T-DNA between the single-stranded scissions was found in a single-stranded linear form (referred to as T-strands). This was most elegantly demonstrated by the finding that this DNA could be size fractionated and transferred to nitrocellulose without any prior denaturation (206). Since only single-stranded DNA binds nitrocellulose under these conditions, this finding suggested that the DNA was single stranded. These sequences could be hybridized by using strand-specific probes complementary to the bottom strand but not the top strand, indicating that only the bottom strand is recovered in single-stranded form. Similarly, an RNA probe could be hybridized to the border DNA even if this DNA was not previously denatured (5).

The VirD2 protein plays an especially noteworthy role in T-DNA processing. VirD2 protein binds tightly to the 5′ end of T-strands (63, 88, 95, 244, 269). Upon extraction with phenol, the VirD2–T-strand complex is found at the phenol-water interphase. This complex survives boiling in 1% SDS and treatment with reducing agents or 6 M urea and is therefore probably caused by a covalent linkage. The carboxy terminal half of VirD2 is dispensable for nicking and covalent linkage (60, 101, 244, 268). A fragment of the VirD2 protein from a nopaline-type Ti plasmid containing the amino-terminal 228 amino acids (of a total length of 447 amino acids) was proficient in nicking, while a fragment of 190 amino acids was deficient (240).

VirD2 has been hypothesized to be a "pilot" protein, which could guide the T-strands to the nucleus (95), possibly by mimicking cellular proteins which contain signals which target them to nuclei. The carboxy terminus of VirD2 contains a sequence similar to a family of nuclear-targeted proteins of animals and yeasts (36, 209). Deletion of 25 codons from the 3′ end of *virD2*, including those encoding this putative pilot sequence, abolishes virulence (211), indicating that this region of the protein plays some essential role in tumorigenesis. To test the hypothesis that this part of VirD2 provides a pilot function, Howard et al. have constructed a gene fusion between *gus* (which encodes β-glucuronidase) and the 3′ end of *virD2*, transiently introduced these constructs into plant protoplasts by electroporation, and observed that the β-glucuronidase activity was localized primarily in the nucleus (96). However, other experiments have indicated that the amino-terminal 292 amino acids of VirD2 could also contain a nuclear-targeting signal. Transgenic plants containing either the native *lacZ* gene or a *virD2::lacZ* translational fusion gene were created (89). Plants containing the native *lacZ* gene contained β-galactosidase localized preferentially in the cytoplasm, while plants containing the hybrid gene contained β-galactosidase localized preferentially in the nucleus. It is therefore possible that the amino and carboxy termini each provide a functional nuclear targeting signal.

If VirD2 does enter the nucleus, it could even play some role in the integration of T-DNA into the plant genome. Supporting this hypothesis is the observation that the central region of the protein has a sequence similar to *E. coli* DNA ligase (259; see reference 99 for the sequence of DNA ligase). The region from residues 140 to 350 of VirD2 is

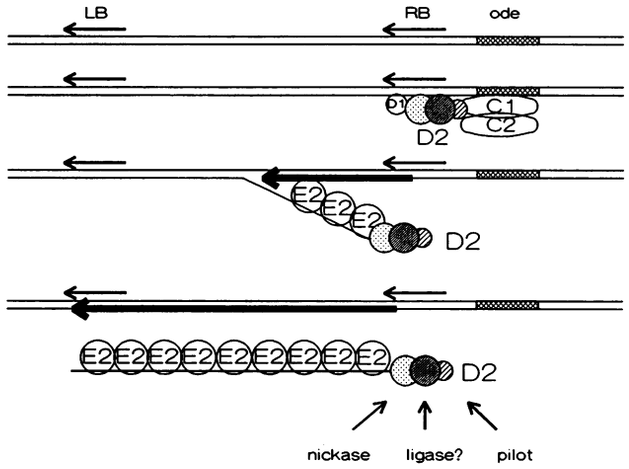

FIG. 5. Proposed mechanism of synthesis of single-stranded T-DNA. LB, RB, and ode refer to the left border, right border, and *overdrive* sites, respectively. Thick arrows indicates newly synthesized DNA which could displace the bottom strand of T-DNA. C1, C2, D1, and D2, represent the products of the *virC1*, *virC2*, *virD1*, and *virD2* genes, respectively.

identical to the amino terminus of ligase at 15% of the positions and has conservative substitutions at 55% of the positions. On the other hand, the VirD2 sequences from the nopaline-type and *A. rhizogenes* plasmids are less similar to DNA ligase, and a lysine residue which is conserved among several ligases is absent in VirD2 (226). A hypothetical domain structure for VirD2 is presented in Fig. 5.

Two other proteins may interact with VirD1 and VirD2 during nicking. The products of VirC1 and VirC2 are thought to bind to the *overdrive* site of octopine-type plasmids and somehow to contribute to T-strand formation (227). Mutations which abolish both VirC proteins do not abolish virulence, but rather attenuate it about 100-fold. Deletions of *overdrive* result in about the same level of attenuation, and, significantly, a strain containing both a *virC* mutation and an *overdrive* mutation is no more deficient in tumorigenesis than a strain containing only one or the other mutation (102). VirC1 protein binds specifically to *overdrive* in a gel retardation assay and can protect this site from nuclease digestion in a footprint assay (227). It has also been reported that VirC proteins stimulate production of T-strands in strains of *E. coli* that express small amounts of the VirD1 and VirD2 proteins, whereas in strains synthesizing high levels of VirD, VirC proteins are not needed for efficient T-strand production (60), suggesting that they could play some ancillary role in DNA unwinding or replacement DNA synthesis. Although one group reported that *virC* mutants are slightly deficient in nicking (228), others reported no deficiency (205). The amino-terminal 60 amino acids of VirC1 show some degree of amino acid sequence similarity to the RepA protein of pTiB6S3 (35% identity and 27% conservative substitutions) and of pRiA4, which are accessory proteins for vegetative DNA replication (259; see reference 215 for RepA sequences).

At least one other protein may bind to the T-strands. The VirE2 product binds to single-stranded DNA (42, 44, 50, 79). Binding has no apparent sequence specificity and is highly cooperative (44, 187). Das and Pazour (51) report that VirE2 does not bind duplex DNA or single-stranded RNA, although Zambryski et al. (273) obtained data that this protein does bind single-stranded RNA. Sufficient VirE2 is present in induced *Agrobacterium* spp. to bind all intracellular T-strands. It has therefore been proposed that VirE2 may bind along the whole length of the T-strand, perhaps to protect it from endonucleases it may encounter during its sojourn from the bacterial cytoplasm to nucleoplasm of the plant cell. Two indirect pieces of evidence could lead one to speculate that VirE1 may play a similar role. The first is that VirE1 stabilizes VirE2 in *E. coli* (139), suggesting that they could form a functional complex. Second, mutations in either *virE2* or *virE1* share the unusual property that their avirulent phenotype is rescuable by coinfection with strains that express *vir* genes but lack T-DNA (42, 159). This suggests either that the VirE proteins can be donated to the *virE* mutant strains and can be recycled or that T-DNA can be transferred from the *virE* mutant to the rescuing strain and from there to the plant. This property is shared by the *virF* product (141, 160), and there are several other examples of complementation of a mutation in one bacterium by a gene in a different bacterium (61, 92, 111, 265; see reference 258 for a more thorough discussion). A model for the formation of T-strands is shown in Fig. 5.

How might this hypothetical T-strand–protein complex traverse the bacterial membranes and peptidoglycan? There is some evidence the *virB* products could create a pore through which T-DNA would pass. *virB* encodes 11 proteins (123, 192, 222, 245, 246), and, significantly, 10 of these have a hydropathy profile which suggests an extracellular or membrane-spanning topology. Three proteins encoded by the 5′ end of *virB* fractionated with the cell envelope (70). Similarly, VirB10 localized to the inner membrane, where it formed either a native oligomer or a complex with other proteins (247). Recently, Vir$^-$ mutations were generated by using Tn*phoA*, a transposon which identifies secreted proteins (132). Many of the mutants obtained by this method were in the *virB* operon, indicating that these proteins are indeed partly or fully secreted (32). Tn*phoA* has also been used to mutagenize *virB* genes specifically, and secreted proteins were again identified (223, 247). In contrast, *virB11* is a hydrophilic protein which binds and hydrolyzes ATP, and is capable of autophosphorylation. It has been hypothesized that it provides a source of energy for the transfer of the DNA-protein complex across the bacterial envelope (41). The predicted protein is homologous to the *Bacillus subtilis comG* ORF1 protein (3). *comG* ORF1 is required for transformation competence and may play a role in the transcriptional regulation of other competence genes. However, there is no evidence that *virB11* encodes a regulatory protein.

Conjugation Model for T-DNA Transfer

A striking similarity between the metabolism of T-DNA and the metabolism of plasmid DNA during bacterial conjugation was first noted by Stachel and Zambryski (208) and by Albright et al. (5) and later expanded upon by Zambryski (270, 271), Ream (173), and others. In both systems, the transferred DNA appears to be a single-stranded molecule with a molecule of the nicking enzyme covalently attached to the 5′ end. In both cases, a single-stranded DNA-binding protein has been implicated (254). Two kinds of evidence support the model that *vir* genes evolved from genes that mediate conjugal transfer (*tra* genes). The first is the finding that the *oriT* site of broad-host-range plasmid RK2 is similar to the T-DNA borders and is nicked at precisely the same position (248). Furthermore, the protein encoded by the *virD4* gene is homologous to the *traD* product of the F

plasmid (259) and to the RK2 *traG* product (248). The F-plasmid TraD protein is thought to be required for DNA synthesis in conjugal donors (116).

The second kind of evidence is the finding that derivatives of the broad-host-range, mobilizable plasmid RSF1010 containing a plant-selectible gene but not containing a T-DNA border have been transferred to plant nuclei and integrated into nuclear DNA (22). Transfer required the RSF1010 *oriT*, at least one of the three *mob* gene products, and *Agrobacterium vir* genes. RSF1010 is not self-transmissible, but is mobilizable by coresident self-transmissible plasmids. Mobilization requires an *oriT* in *cis* and the products of *mob* genes supplied in *cis* or in *trans*. These *mob* gene products constitute a site-specific endonuclease which causes nicks at *oriT*. All other transfer proteins are provided by the coresident transmissible plasmids. The finding that *vir* genes are functionally interchangeable with bacterial conjugal transfer genes indicates a very strong similarity between the two. Very recently, it has been demonstrated that two different conjugal plasmids (RK2 and F) are able to transfer from *E. coli* to the yeast *Saccharomyces cerevisiae* (87), indicating that conjugal transfer systems may be more promiscuous than previously thought.

As the similarities between T-DNA transfer and conjugal plasmid transfer have been noted in other recent reviews, it may be useful here to emphasize the differences between the two systems. One major difference is the presence of single-stranded, linear T-DNA in the donor before transfer. In the bacterial conjugation systems, the transferred DNA is single stranded only transiently during transfer and a complementary strand is quickly (although not obligately) synthesized in both donor and recipient (254). Another major difference concerns the fate of the transferred DNA. Conjugally transferred DNA recircularizes in the recipient to create an autonomously replicating plasmid indistinguishable from that in the donor. This DNA may also be integrated into recipient DNA by homologous recombination. In contrast, *Agrobacterium* T-DNA is covalently integrated by illegitimate recombination into plant genomic DNA. Therefore, even though the two systems may have had a common ancestor, there clearly are important differences, and one must use caution when applying information from one system to predict properties of the other.

Other models have been put forth to describe the transfer of T-DNA; most of these invoke the double-stranded breakage and circularization of the DNA prior to transfer. As mentioned above, double-stranded breaks at each border have been reported by several groups (210, 233). Joining of these borders by Campbell recombination has been inferred from indirect genetic observations but never observed by physical methods (120). Covalently joined left and right borders, forming "hybrid" or "joint" borders, have been recovered in both *A. tumefaciens* and *E. coli* (6, 120, 131, 224). It cannot be denied that these circular molecules are formed within bacterial cells, but their existence does not prove that they represent transfer intermediates rather than the products of some side reaction. An alternative explanation for their occurrence is that the nicks catalyzed by VirD1 and VirD2 provide substrates for host-mediated homologous recombination. According to this model, such circular molecules might not be transferable to plants.

Bakkeren et al. (12) sought to determine whether circular T-DNA molecules can enter plant nuclei; they did so by recovering such molecules from plants by using the process of "agroinfection." An *Agrobacterium* strain containing one genome length of cauliflower mosaic virus inserted between T-DNA borders was constructed. This strain was used to transfer the viral DNA to plants. This DNA then formed circular infectious agents. This procedure provides a selective pressure for circularization of the transferred DNA at sites at or close to the left and right T-DNA borders. Infectious virus is obtained whether circularization occurs in the bacterium or in the plant. Importantly, agroinfection does not impose any selection for DNA integration. If the true transfer intermediate were a circular molecule containing a hybrid border, the agroinfection assay should provide a way to recover the progeny of these molecules without any alterations that normally accompany the integration step. Viruses recovered from plant tissues did not contain perfect hybrid borders. Bakkeren et al. (12) concluded that the circular molecules containing hybrid borders that had been isolated from bacteria in earlier studies do not represent transfer intermediates.

Integration of T-DNA into the Plant Genome

In contrast to the many recent advances in our understanding of T-DNA metabolism in *A. tumefaciens*, very little is known about the fate of this DNA in plant cytoplasm and nucleoplasm. In one study, DNA was isolated from *Petunia* protoplasts at intervals up to 24 h after infection by *A. tumefaciens* and probed for T-DNA, other Ti-plasmid DNA, and bacterial chromosomal DNA (237). It was concluded that other Ti-plasmid DNA in addition to the T-DNA was transferred and that most of the DNA transferred to plants was degraded. This potentially powerful approach has not been reported by others, largely because of the technical difficulties of recovering bacterial DNA from plant tissues free of contaminating nontransferred DNA. A large number of studies have been undertaken to determine the number and location of T-DNA inserts in mature tumors. One or more than one T-DNA molecules can be transferred and integrated (43, 108, 201, 221, 229). In bacterial strains which contain two T-DNAs, one or both may be transferred to a given transformed plant cell. In cells containing more than one T-DNA, the extra copies are sometimes tightly linked to each other and sometimes dispersed throughout the plant genome. The copies that are linked can be in tandem or inverted orientation (43, 108).

The DNA sequences of several T-DNA-host DNA junctions has been determined (93, 193, 266, 274). Gheysen et al. (78) pointed out that these junctions, in general, appear more variable than the junctions created by insertions of transposons, retroviruses, or retrotransposons. Rather, they are similar to the junctions created by insertions of non-retrotransforming viruses such as simian virus 40 or adenovirus. In general terms, it seems that the right junction is somewhat less variable than the left. The right end of the T-strand created in the bacterium contains 3 bp from the right border and a covalently bound VirD2 protein, while the left end contains 22 bp from the left border and is free of covalently bound protein (see above). In most cases the right junction of the DNA includes all or almost all bases of the T-strand (reviewed in reference 271). In contrast, the left end of the DNA after transfer usually lacks some of the sequences of the left end of the T-strand. The number of missing bases ranges from a few up to 100 bp. It is tempting to speculate that T-strand integration, like its formation, could be initiated at its right end and could even be mediated in part by VirD2.

The target sites of T-DNA integration before and after integration have been sequenced in three studies (78, 134,

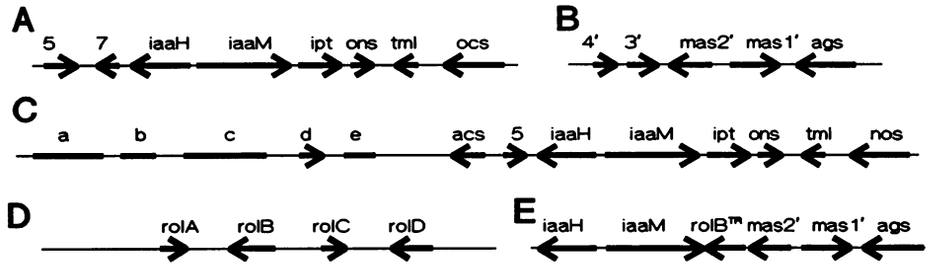

FIG. 6. Genetic organization of the T-DNA of two *A. tumefaciens* strains and of *A. rhizogenes*. (A) T_L-DNA of plasmids similar to pTiA6. (B) T_R-DNA of plasmids similar to pTiA6. (C) T-DNA of strains similar to pTiC58. (D) T_L-DNA of *A. rhizogenes* plasmid pRiA4. (E) T_L-DNA of *A. rhizogenes* plasmid pRiA4. Genes are described in Table 3. For genes having more than one name, the most descriptive name is shown. Additional transcriptional units in the *A. rhizogenes* T_L-DNA have been observed but not localized.

138). The first study examined a single integration event of T-DNA into the tobacco genome. This insertion caused a duplication of 158 bp of host DNA, a small deletion, a translocation, and several single-base-pair transitions. In a second study, seven different integration events into the genome of *Arabadopsis thaliana* were examined (138). As in earlier studies, the left junctions between T-DNA and plant DNA showed less precision than the right junctions. At the left junction, only two insertions contained all sequences found on the T-strand, including the 22 bases derived from the left border. The other insertions lacked either 7, 16, 20, 31, or 100 bases from the left end of the T-strand. At the right junction, four of the T-DNA inserts contained all sequences found at the right end of T-strands, including the three bases derived from the right border, while two inserts lacked 2 bases and one insert lacked 32 bases of T-strand DNA. In all cases, the target sequences suffered small (29- to 73-base) deletions. In three cases, either 9, 15, or 19 bases of "filler DNA" separating T-DNA and host DNA were found. Perhaps most significantly, in four inserts a limited sequence identity (5 to 7 bases) was found between the extreme ends of the inserted T-DNA and the corresponding target sequences. These results suggest that integration may involve a process of illegitimate recombination (129) involving either the extreme ends of the T-DNA or bases near the extreme ends. The observation that the right junction usually contains all sequences present on the T-strand suggests that this end remains protected, probably by VirD2, up to the moment of integration. The third study comparing T-DNA junctions with target sequences, like the first study, examined a single insertion into the tobacco genome (134). In this study, the junctions between T-DNA and host DNA occurred 350 bp internal to the left border and 7.3 kb internal to the right border. As in the second study, a small deletion (23 bases) occurred at the target site and the target site contained limited but significant sequence similarity to the boundaries of the inserted T-DNA sequences.

Expression of Transferred Genes

Figure 6 and Table 3 show the genetic organization of the T-DNA of the octopine-type and nopaline-type strains of *A. tumefaciens* and the T-DNA of an *A. rhizogenes* strain. Although there is evidence that the T-DNA genes were not evolutionarily derived from plants, they have nevertheless evolved eukaryoticlike regulatory sites. Transcription of all T-DNA genes is inhibited by α-amanitin, an inhibitor of RNA polymerase II, indicating that their expression, like other protein-encoding genes, depends upon this RNA polymerase (256). Inspection of the DNA sequence of these promoters generally reveals CAAT and TATA boxes typical of plant promoters (reviewed in references 77 and 157). Furthermore, the mRNA from these genes contain eukaryotic poly(A) tails and polyadenylation sites have been identified in several of these genes. A final similarity between T-DNA genes and plant genes is that at least some transferred genes contain upstream activating sites that are similar to eukaryotic transcriptional enhancers (66, 194). On the other hand, unlike most plant genes, no T-DNA gene contains an intron (157).

Most or all of the genes in T-DNA can be categorized as being involved either in upsetting the normal balance of

TABLE 3. Descriptions of genes transferred to plant cells by Ti or Ri plasmids

Locus	Alternate names	Protein function	Role in tumorigenesis	References
iaaM	*tms1*, *shi*, transcript 1	Tryptophan monooxygenase	Auxin synthesis	186, 219
iaaH	*tms2*, *shi*, transcript 2	Indoleacetamide hydrolase	Auxin synthesis	186, 219
ipt	*tmr*, *roi*, transcript 4	Isopentenyl transferase	Cytokinin synthesis	2, 23
5		?	Tumorigenesis	124, 198, 225
tml	transcript 6b	?	Tumorigenesis	198
rolA–rolD		?	Root formation	33, 185, 195, 199, 252
rolB(Tr)		?	Root formation?	18
ocs		Octopine synthase	Opine synthesis	54
nos		Nopaline synthase	Opine synthesis	15
acs		Agrocinopine synthase	Opine synthesis	86, 107
mas2'	2'	Mannopine synthesis	Opine synthesis	19, 69, 183
mas1'	1'	Mannopine synthesis	Opine synthesis	19, 69, 183
ags'	0'	Agropine synthesis	Opine synthesis	19, 69, 183
ons	transcript 6a	Permease	Opine secretion	147

phytohormones of the host cell or in the production of opines. Two genes have been implicated in the overproduction of auxin. *iaaM* and *iaaH* (also known as transcripts 1 and 2 or as *tms1* and *tms2*) encode proteins that convert tryptophan to indoleacetic acid via indolacetamide (186, 219). The *ipt* gene (also known as transcript 3 or *tmr*) catalyzes the condensation of isopentenyl pyrophosphate and adenosine monophosphate to form isopentenyl-AMP, which is converted by host enzymes to *trans*-zeatin and *trans*-ribosylzeatin (2, 23). In addition, two other genes play a poorly characterized ancillary role in tumor formation. *tml* and transcript 5, although not oncogenic alone, appear to modulate tumorigenicity when present in combination with other oncogenes (124, 198, 225).

In contrast to *A. tumefaciens*, *A. rhizogenes* appears to use a different strategy for oncogenesis. The genes most important for root proliferation appear to be the four *rol* (root locus [33, 252]) genes. These genes do not change the levels of endogenous phytohormones, but, rather, one or more of them appear to increase the sensitivity of transformed roots to exogenously applied auxins (190). They presumably also sensitize host cells to endogenous auxins, and this is probably the underlying mechanism of hairy-root formation. Fertile plants can be regenerated from transformed roots, and these plants have characteristic properties, including wrinkled leaves, short internodes, reduced geotropism of roots, and floral hyperstyly. Certain features of the hairy-root syndrome appear to be attributable to individual *rol* genes (185, 195, 199). Transgenic plants containing just *rolA* exhibit wrinkled leaves (185, 195), whereas plants containing just *rolB* exhibit altered flower morphology, heterostyly, and increased numbers of adventitious roots on stems. Plants containing just *rolC* show reduced apical dominance and internodal distance, altered leaf morphology, small flowers, and reduced seed production (185). The mechanisms by which they do this are completely unknown and will certainly be the source of exciting research in coming years.

In addition to *rol* genes, some strains of *A. rhizogenes* do transfer auxin biosynthetic genes, whereas others do not (252). The transfer of these genes alone results in weak root formation. The transfer of *rol* genes without *iaa* genes (whether by mutation of *iaa* genes or by using naturally occurring strains that do not contain them) results in tumorigenesis only on a restricted number of hosts and on a more restricted number of plant surfaces (236). In particular, *iaa* mutants caused root formation only on the apical surface of carrot root slices, where auxin concentrations are naturally higher than on the basal surface. Addition of the *iaa* genes to these bacteria, or addition of auxin to the inoculation site, resulted in tumorigenesis of the basal surface (34). The fact that adding auxin to the wound site allows root formation means that it is needed only transiently. No strain of *A. rhizogenes* transfers an *ipt* gene, although at least one strain does have a homologous gene which is not transferred (174).

At least some *rol* genes and *iaa* genes are induced by auxin (27, 137). These genes are spatially regulated, expressed only at sites known to have high auxin levels, chiefly at apical meristems. This results in a form of signal amplification; at sites of endogenous auxin biosynthesis, the transformed cells containing *iaa* genes synthesize additional auxin, while transformed cells containing *rol* genes become more sensitive to these auxins. Transformed cells containing both types of genes suffer both an increased synthesis and an increased sensitivity. These observations go a long way toward explaining the hairy-root tumor morphology caused by *A. rhizogenes*.

In addition to carrying oncogenes, T-DNAs encode opine biosynthetic enzymes. Opines of the octopine family are synthesized by the *ocs* product in a reductive condensation of pyruvate with arginine, ornithine, lysine, or histidine. Opines of the nopaline family are synthesized by the *nos* product in a similar condensation involving α-ketoglutarate and either arginine or ornithine (unlike the Ocs enzyme, histidine and lysine are not substrates for Nos [169]). Leucinopine and succinamopine have structures similar to nopaline (35, 40) although their biosynthetic precursors have not been identified. Mannityl opines are made by condensation of glucose with glutamine or glutamic acid followed by reduction of the sugar to form mannopine and mannopinic acid, respectively. Mannopine is enzymatically lactonized to make agropine and also undergoes spontaneous lactonization to form agropinic acid (56, 57, 69). In plasmids similar to A6, the products of genes 2′ and 1′ (located in the T_R region) direct the first two reactions, while the 0′ genes encodes the lactonizing activity (69, 183). Agrocinopines A and B are made by condensation of arabinose with sucrose or fructose (181). A separate transferred gene (*ons*) appears to mediate the excretion of at least some of these opines from plant cells (147).

Release from Bacteria of Other Signal Molecules

In addition to transferring DNA to plants, some or all strains release low-molecular-weight, diffusible compounds. *A. rhizogenes* and nopaline-type strains of *A. tumefaciens* contain a nontransferred cytokinin biosynthetic gene called *tzs* (for trans-zeatin synthase) which is homologous to the transferred *ipt* gene. This gene lies to the left of the *vir* gene cluster, and its expression is regulated by the *virA* and *virG* products (106). This means that the gene is induced at the outset of infection, resulting in a burst of cytokinin biosynthesis at the time that T-DNA is transferred. The significance of this is unknown, but since cytokinins stimulate cell division, they ought to weaken the plant cell wall and stimulate DNA synthesis, both of which could facilitate T-DNA transfer or integration. Mutations in this gene have not been described, but octopine-type Ti plasmids do not have this gene, so it must be dispensable for pathogenicity. Alternatively, this gene could be expressed continuously in bacteria colonizing the tumor and might materially affect the level of cytokinins.

It has also been hypothesized that *Agrobacterium* spp. can release auxin to plant cells. A localized tissue necrosis called the hypersensitive response normally occurs in tobacco in response to inoculation by *Pseudomonas syringae* pv. phaseolicola. The hypersensitive response has been reported to be prevented by preinoculation of the wound site by *A. tumefaciens* (176). Analysis of *Agrobacterium* mutants indicated that the *tms* locus was required and sufficient. This locus is normally transferred to plant cells, where it directs the overproduction of auxin (see above); however, the genes necessary for transfer were not needed, so transfer must not be required. It is as though expression of *tms* in bacteria is necessary and sufficient to prevent the hypersensitive response. Another bacterium known to produce and secrete large amounts of auxin, *Pseudomonas savastanoi*, was also effective at preventing the hypersensitive response.

A. tumefaciens bv. 3 (recently renamed *A. vitis* [158]), commonly isolated from grapevines, is able to release a pectolytic enzyme (177). These strains are unique among

Agrobacterium isolates in that they can cause a root decay on susceptible strains of grapevine (24). This disease does not require the Ti plasmid. The gene encoding this pectinase has been disrupted by transposon mutagenesis. The resulting mutant was unable to incite root rot and was somewhat attenuated in virulence (177).

CONCLUDING REMARKS

Despite the fact that more than 80 years have passed since Smith and Townsend applied Koch's postulates to crown gall disease (197), most of the seminal discoveries about this disease have been made in the past 5 to 10 years. We now have at least some insight about how *Agrobacterium* spp. perceive low-molecular-weight compounds which diffuse from plant wounds. Yet, as so often happens, with each insight come new questions. We do not know whether VirA or some other protein contains the binding site for phenolic inducers. We do not understand the low pH optimum for *vir* gene induction. We do not know how inducing compounds alter the ability of VirA to phosphorylate VirG, since the regulated step could be autophosphorylation of VirA, phosphate transfer to VirG, or phosphate removal from VirG. Furthermore, VirA and VirG fail to activate *vir* genes in *E. coli* (259), suggesting that additional, undiscovered proteins may be required. We also do not understand the induction of *virG* by acidic environments, and we do not understand the mechanism or the adaptive significance of *virC* and *virD* repression by the *ros* product. Finally, we have much to learn about the induction of chromosomal genes by plant-released compounds.

We have learned a lot about the processing of T-DNA within bacteria, although some controversy remains about whether T-DNA is transferred as a single-stranded linear molecule or in some other form. The nicking reaction catalyzed by VirD1 and VirD2 has not been reproducibly observed in vitro, so the biochemistry of this reaction remains uncharacterized. The interactions between *virC*-encoded proteins and *overdrive* need further study, especially since the nopaline-type plasmids do not have such sites but do have *virC* genes. The existence of a T-complex consisting of T-DNA, VirD2, and VirE2 is still based on rather indirect evidence, and has not been observed directly. Future studies will also place more emphasis on events that occur outside the bacterial envelope.

We now understand the mechanism of action of certain transferred genes. The discoveries that T-DNA genes direct the biosynthesis of auxin, cytokinin, and opines are probably the most significant insights made to date. Despite this, the functions of other T-DNA genes remain enigmatic. Among these, a better understanding of the *A. rhizogenes rol* genes will certainly provide important insights about plant growth and development. Finally, we understand almost nothing about *Agrobacterium*-plant interactions that follow the formation of crown gall tumors. *Agrobacterium* strains may have specific strategies for colonizing the specialized ecological niches that they create. Clearly, much work remains to be done.

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